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Title

New insights into the genetics of primary open-angle glaucoma based on meta-analyses of intraocular pressure and optic disc characteristics.

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Abstract

Primary open-angle glaucoma (POAG), the most common optic neuropathy, is a highly heritable disease. Intraocular pressure (IOP) and optic nerve head characteristics are used clinically to predict POAG risk. We conducted a genome-wide association meta-analysis of IOP and optic disc parameters and validated our findings in POAG cases. We identified 21 new genomic regions associated with optic nerve head variation and with IOP. Several genomic regions affect both IOP and the optic disc and we found that pathways involved in these endophenotypes are not entirely distinct as assumed. We further identified a novel association between *CDKN1A* and POAG. Using a zebrafish model we demonstrate that *six6b* (associated with POAG and optic nerve head variation) alters the expression of *cdkn1a*.

Author Summary

Glaucoma is a progressive eye disease that damages the optic nerve and is a leading cause of irreversible blindness worldwide. In this study we conducted the largest genome-wide association meta-analysis of the measurements used clinically to predict glaucoma risk. We identified 21 new genomic regions associated with these measurements and we found that *CDKN1A* influences glaucoma risk. Furthermore, we explored the interaction between *CDKN1A* and *SIX6* (another glaucoma gene) in zebrafish. We identify genetic variants associated with more than one of the measurements analyzed, and we found new and well-known pathways. Our findings provide new insights into the pathways and genes that underlie glaucoma and the measurements used clinically to predict glaucoma risk.

Introduction

In primary open-angle glaucoma (POAG), loss of retinal ganglion cells and nerve fibers manifests itself clinically as optic nerve damage, which leads to visual field loss and, eventually, blindness. The optic nerve damage is characterized by an increase in cup size, the central area of the optic nerve head (or optic disc). This damage can be quantified by the vertical cup-disc ratio (VCDR), comparing the vertical diameter of the cup with the vertical diameter of the total optic disc.

Elevated intraocular pressure (IOP) is a well-recognized risk factor and current POAG therapies lower IOP by various mechanisms. Sib relative risk analyses suggest that POAG is highly heritable[1] and several genome-wide association studies (GWAS) have identified new POAG genes by examining POAG directly or studying endophenotypes like VCDR and IOP[2-11]. Several genes associated with VCDR and IOP - *CDKN2B-AS1*, *SIX6* (VCDR); and *CAV1/CAV2*, *TMCO1*, *ABCA1* and *ARHGEF12* (IOP) - are highly significantly associated with POAG. Notably, no genes have been significantly (genome-wide) associated with both VCDR and IOP. Charlesworth et al. previously found a genetic correlation between VCDR and IOP (RhoG = 0.45, P = 0.0012), however, genes underlying this relationship have not yet been identified[12].

The aims of this study were to (1) identify new genes associated with the POAG endophenotypes IOP, VCDR, cup area, and disc area, and ultimately POAG, using the 1000 Genomes imputations reference panel, and (2) investigate the genetic overlap between the different endophenotypes. To accomplish these aims we performed a meta-analysis of GWAS of these four traits within the International Glaucoma Genetics Consortium (IGGC).

Results

Intraocular pressure

After removal of single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) < 0.01 and low imputation quality, approximately 8 million SNPs were included. Whilst the analysis of individuals of European descent yielded no novel associations, combined analysis of individuals of European and Asian descent (n = 37,930, λ = 1.07; **S1a, S1b and S2b Figs**), yielded nine genomic regions reaching genome-wide significance, of which eight genomic regions were already known (**S1a, S1b and S2b Figs**, **S3 Table**)[4, 6, 8]. The peak SNP in the new genomic region was rs55796939 on chromosome 11q25 near *ADAMTS8* (**S3 and S4 Figs**).

Vertical cup-disc ratio

In the meta-analysis of individuals of European descent (n = 23,899, λ = 1.10), 21 genomic regions were genome-wide significant (**S5a** and **S6a Figs**, **S4 Table**). Five genomic regions were novel (near to the genes *RPE65* on chr. 1p31, *F5* on chr. 1q23, *PDZD2* on chr. 5p13.3, *RREB1* on chr. 6p25, and *DGKB* on chr. 7p21.2) (**S7** and **S8 Figs**); the other genomic regions have been previously associated with VCDR or cup area, two highly correlated traits[13-15]. Of the five novel genomic regions, *RREB1* (p-value = 4.00×10^{-3}) was nominally significant in the analysis of individuals of Asian descent (n= 8,373, λ = 1.02). In the combined analysis (n = 32,272, λ = 1.08), another four novel genomic regions, near to the genes *VCAN* on chr. 5q14.3, *PSCA* on chr. 8q24.2, *ENO4* on chr. 10q25.3, and *RBM23* on chr. 14q11.2 (**S5b** and **S6b Figs**), were genome-wide significant leading to a total of nine (5+4) novel genomic regions associated with VCDR. Of these novel genomic regions, *F5* has been associated with disc area previously[15]. Disc area influences the VCDR[16], and therefore we corrected VCDR for disc area in a secondary analysis. After correction for disc area, the β (p-value) decreased from -0.007

(2.48 x 10^{-9}) to -0.002 (5.60 x 10^{-2}) in the subset with disc area available, suggesting that *F5* acts primarily on disc area and secondary to VCDR through its relation to disc area.

Cup area

The meta-analysis of individuals of European descent (n = 22,489, λ = 1.09) yielded 20 genome-wide significant regions of which 17 regions were already implicated for cup area or VCDR (**S9a** and **S10a Figs**, **S5 Table**)[14, 15]. There were three novel associations on chr. 1q42.11 near *CDC42BPA*, chr. 8q21.11 near *CRISPLD1*, and on chr. 15q26.3 near *FAM169B* (**S11** and **S12 Figs**). *CDC42BPA* has previously been associated with disc area and the fact that the association with cup area adjusting for disc area is genome wide significant suggests an independent effect on cup area. In the combined analysis of European and Asian individuals (n = 29,828, λ = 1.08, **S9b** and **S10b Figs**) all loci except *FAM169B* remained genome-wide significant, and there were two additional genome-wide significant SNPs at chr. 6p21.2 (*CDKN1A*) and chr. 9q34.2 (*ABO;* previously associated to IOP).

Disc area

The meta-analysis of individuals of European descent (n = 22,504, λ = 1.09) resulted in 13 genome-wide significant regions, of which two were not previously associated with disc area: *UGT8* on chr. 4q26 and *CTNNA3* on chr. 10q22.2 (**S13a**, **S14a**, **S15**, **S16 Figs**, and **S6 Table**). These SNPs were not significant in the meta-analysis of individuals of Asian descent (n = 7,307, λ = 1.03). An additional four SNPs reached genome-wide significance in the combined meta-analysis (n = 29,811, λ = 1.09): *PRDM16* on chr. 1p36.23-p33, *GADD45A* on chr. 1p31.2, *VGLL4* on chr. 3p25.3, and *ASB7* on chr. 15q26.3 (**S13b** and **S14b Figs**).

Characterization of the lead association signals

In total, 86 SNPs were associated with one or more of the above endophenotypes. Functional characterization of the 86 SNPs was performed using a range of bioinformatics tools (see **Methods**). In total, 1435 variants in linkage disequilibrium (LD) with the 86 lead SNPs (R^2 >0.8) were examined for functional annotation. Overall, 59% (51/86) of the associated loci are in LD with variants located in regulatory regions according to the ENCODE data (e.g. DNase I hypersensitive sites, transcription factor binding sites and motifs; see **S7 Table**). We investigated the expression levels of the identified candidate genes using the UniGene database[17]. Of all reviewed genes, *CDKN1A, PAX6* and *DUSP1* showed the highest number of transcripts per million in the eye (**S8 Table**). According to the Ocular Tissue database[18], *CDKN1A* is highly expressed in the optic nerve head, as well as *DUSP1,* which also shows high expression in the trabecular meshwork. Both genes were associated with optic nerve head parameters. *PAX6* is highly expressed in the ciliary body and retina, in this study we found it associated with disc area. Other highly expressed genes in the optic nerve include *EFEMP1* and *ABI3BP*, which are associated with cup area and disc area, respectively (**S9 Table**).

Gene-based test

To identify new loci not found through per-SNP tests, we performed gene-based testing using VEGAS2. Reflecting the smaller number of tests, our gene-based significance threshold is $P_{gene\text{-}based} < 0.05/24{,}769 = 2.02 \times 10^{-6}$ (24,769 genes tested). Using the gene-based test we found several novel loci (**S10 Table**). *C9* was significantly associated with IOP (p-value 1.61×10^{-6}); *RARB* (p-value 1.86×10^{-6}) and *HORMAD2-AS1* (p-value 1.04×10^{-6}) were associated with VCDR. These genes were previously associated with disc area, so the novel associations with VCDR could possibly be driven by the influence of disc area on VCDR[15]. In the cup area analysis, the genes *LRP10* (p-value 1.20×10^{-6}) and *REM2* (p-value 1.55×10^{-6}), and *THSD4* (p-value 5.44×10^{8}) were significantly associated. The first two genes are located near to *RBM23*, which was significant in the per-SNP test. *THSD4* is located near to *KPNB1*, which was associated with VCDR in our previous meta-analysis[14]. In the disc area

analysis we found two genes that were significantly associated with disc area: *ANKRA2* (p-value 8.42 x 10^{-7}) and *LOC149950* (p-value 3.87 x 10^{-7}).

Characterizing the overlap in biological pathways involved in glaucoma endophenotypes

In total, 86 SNPs were associated with one or more of the above endophenotypes. The effect estimates and p-values of these SNPs for all four endophenotypes are shown in **Table 1-3**. *ADAMTS8* (IOP and VCDR, Table 1 and Table) and *ABO* (IOP and cup area, Table 1 and Table) were genome-wide significantly associated with two traits. Of note is that there were different variants involved in *ADAMTS8*: rs55796939 for IOP and rs4936099 for VCDR (r2=0.03 between these SNPs in 1000G European samples). **Figure 1** shows the overlap in associations across endophenotypes – we depict annotated genes for which at least one SNP was genome-wide significant in at least one trait. Overlap is defined as nominal significance or stronger for the second trait. The figure shows as expected a strong overlap in variants associated to disc area, cup area and VCDR. Further, overlap is noted in genes associated to IOP, cup area and VCDR.

Table 1. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with IOP and show and association with vertical cup-disc ratio.

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs10918274	*TMCO1*	t/c	0.26	0.04	**3.40E-12**	0.005	0.002	8.60E-03	0.010	0.003	2.20E-03	-0.001	0.006	9.13E-01
rs7635832	*FNDC3B*	g/t	-0.22	0.03	**3.84E-13**	-0.001	0.001	3.33E-01	-0.004	0.003	1.22E-01	0.002	0.005	7.12E-01
rs10281637	*CAV1/CAV2*	c/t	0.20	0.03	**2.53E-13**	0.004	0.001	4.97E-03	0.006	0.003	1.13E-02	-0.003	0.005	5.81E-01
8:78380944	*PKIA*	i/r	1.00	0.17	**6.06E-09**	0.000	0.010	9.77E-01	-0.017	0.017	3.23E-01	0.018	0.031	5.50E-01
rs7815043	*PKIA*	c/t	0.10	0.03	**3.64E-05**	-0.001	0.001	3.23E-01	-0.001	0.002	8.24E-01	-0.002	0.004	5.65E-01
rs7944735	*Many genes*	c/g	0.20	0.03	**3.97E-11**	0.001	0.001	4.53E-01	0.006	0.003	2.97E-02	0.000	0.005	9.73E-01
11:120357425	*ARHGEF12*	d/r	0.18	0.03	**1.54E-09**	0.001	0.001	5.70E-01	0.001	0.003	6.27E-01	0.001	0.005	8.20E-01
rs12794618	*ARHGEF12*	c/t	0.17	0.03	**6.72E-09**	0.001	0.001	3.77E-01	0.002	0.003	4.62E-01	0.004	0.005	4.24E-01
rs55796939	*ADAMTS8*	t/c	0.36	0.06	**1.92E-08**	0.003	0.003	3.77E-01	0.006	0.006	3.06E-01	-0.003	0.010	8.00E-01
rs2472496	*ABCA1*	g/a	-0.17	0.02	**1.47E-13**	0.005	0.001	**5.69E-05**	0.010	0.002	**6.40E-07**	0.003	0.004	4.71E-01
rs8176741	*ABO1*	a/g	0.24	0.04	**2.55E-10**	0.007	0.002	**4.06E-05**	0.019	0.003	**5.65E-08**	0.004	0.006	5.25E-01
rs9913911	*GAS7*	g/a	-0.17	0.02	**4.95E-12**	-0.006	0.001	**1.62E-07**	-0.008	0.002	**1.79E-04**	-0.001	0.004	8.45E-01

For these SNPs, the associations with the other traits are also included. SNPs that are Bonferroni significantly associated with other traits are shown in bold (p-value < 5.31×10^{-4}; 0.05/94). In the first rows, the SNPs genome-wide significantly associated with intraocular pressure (IOP) are shown. Next, the SNPs associated with IOP, vertical cup-disc ratio (VCDR), and cup area are shown. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Table 2a. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with vertical cup-disc ratio and show an association with cup area and disc area

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs6804624	*COL8A1*	c/t	-0.01	0.02	6.63E-01	0.008	0.001	**4.69E-12**	0.013	0.002	**1.66E-08**	0.020	0.004	**4.93E-07**
rs7916697	*ATOH7*	a/g	0.01	0.03	7.39E-01	-0.018	0.001	**5.22E-46**	-0.017	0.002	**7.42E-13**	-0.094	0.004	**7.36E-110**
10:96008348	*PLCE1*	d/r	0.01	0.03	5.81E-01	0.007	0.001	**3.50E-08**	0.013	0.002	**1.11E-08**	0.015	0.004	**1.61E-04**
rs324780	*TMTC2*	g/a	0.03	0.02	2.85E-01	-0.011	0.001	**3.08E-23**	-0.016	0.002	**7.62E-14**	-0.029	0.004	**8.39E-14**
rs4299136	*ASB7*	c/g	-0.03	0.03	4.13E-01	0.010	0.002	**1.72E-12**	0.018	0.003	**3.02E-10**	0.024	0.005	**2.39E-06**
16:51461915	*SALL1*	r/i	0.02	0.03	4.23E-01	0.010	0.001	**1.45E-13**	0.013	0.003	**5.18E-07**	0.032	0.005	**5.19E-13**
rs4784295	*SALL1*	c/g	0.02	0.03	5.61E-01	0.010	0.001	**2.22E-13**	0.013	0.002	**1.19E-07**	0.031	0.004	**1.99E-12**
rs5752773	*CHEK2*	g/c	0.01	0.03	6.89E-01	-0.012	0.001	**7.90E-21**	-0.024	0.003	**1.25E-21**	-0.024	0.004	**5.28E-08**
rs2092172	*CARD10*	a/g	0.00	0.03	8.88E-01	0.009	0.001	**1.92E-12**	0.011	0.003	**2.91E-05**	0.032	0.005	**3.84E-12**
rs7717697	*VCAN*	c/t	0.01	0.02	7.27E-01	-0.007	0.001	**4.39E-09**	-0.009	0.002	**1.12E-05**	-0.018	0.004	**2.16E-06**
rs1681739	*ENO4*	t/c	0.03	0.02	2.19E-01	0.006	0.001	**2.12E-08**	0.011	0.002	**3.32E-07**	0.019	0.004	**1.04E-06**
rs60779155	*ASB7*	a/g	-0.02	0.03	6.57E-01	0.010	0.002	**2.67E-10**	0.019	0.003	**2.92E-09**	0.030	0.005	**3.28E-08**
rs1830890	*PLCE1*	g/a	0.01	0.02	8.27E-01	0.006	0.001	**2.49E-08**	0.012	0.002	**6.52E-08**	0.014	0.004	**3.60E-04**
rs482507	*TMTC2*	c/t	0.02	0.02	3.52E-01	-0.011	0.001	**1.03E-19**	-0.017	0.002	**1.15E-14**	-0.030	0.004	**5.03E-14**
rs4436712	*SIX6*	t/g	-0.04	0.02	1.40E-01	0.009	0.001	**3.58E-14**	0.025	0.002	**1.88E-30**	-0.018	0.004	**3.71E-06**
rs738722	*CHEK2*	t/c	0.02	0.03	3.61E-01	-0.012	0.001	**2.78E-20**	-0.024	0.003	**2.35E-22**	-0.021	0.004	**1.59E-06**
rs2684249	*HSF2*	c/t	0.03	0.02	2.10E-01	-0.006	0.001	**1.47E-07**	-0.012	0.002	**1.96E-08**	-0.015	0.004	**9.56E-05**
rs34222435	*ASB7*	t/c	-0.03	0.03	3.77E-01	0.010	0.002	**1.95E-12**	0.019	0.003	**7.65E-11**	0.025	0.005	**1.29E-06**

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs7916410	*ATOH7*	t/c	0.00	0.03	9.76E-01	-0.018	0.001	**2.23E-46**	-0.017	0.002	**3.57E-12**	-0.097	0.004	**1.97E-114**
rs442376	*TMTC2*	c/t	-0.03	0.03	3.15E-01	0.011	0.001	**6.80E-18**	0.017	0.002	**1.59E-12**	0.032	0.004	**9.79E-15**
rs1345467	*SALL1*	g/a	0.01	0.03	6.55E-01	0.009	0.001	**2.91E-12**	0.012	0.003	**8.53E-07**	0.032	0.004	**1.19E-13**
rs5762752	*CHEK2*	c/g	0.01	0.03	6.58E-01	-0.011	0.001	**2.29E-18**	-0.021	0.002	**2.58E-19**	-0.023	0.004	**1.17E-08**
rs11129176	*RARB*	a/g	0.02	0.03	4.11E-01	0.005	0.001	**3.14E-05**	0.010	0.002	**8.59E-06**	0.023	0.004	**1.02E-08**
rs1997404	*COL8A1*	g/t	-0.02	0.03	3.28E-01	0.008	0.001	**1.36E-11**	0.013	0.002	**6.58E-08**	0.024	0.004	**7.31E-09**
rs34935520	*SIX6*	g/a	-0.04	0.02	1.09E-01	0.009	0.001	**5.41E-14**	0.025	0.002	**9.85E-30**	-0.022	0.004	**2.39E-08**

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with vertical cup-disc ratio that are Bonferroni significantly associated with cup area or disc area are shown in bold (p-value < 5.31×10^{-4}; 0.05/94).Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Table2b. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with vertical cup-disc ratio and show an association with cup area

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs1925953	*RPE65*	t/a	-0.02	0.02	3.13E-01	0.006	0.001	**1.10E-07**	0.010	0.002	**1.20E-05**	0.006	0.004	9.98E-02
rs72759609	*PDZD2*	c/t	-0.04	0.05	3.38E-01	-0.012	0.002	**5.21E-09**	-0.020	0.004	**1.23E-06**	-0.021	0.008	4.69E-03
rs114503346	*DUSP1*	t/c	-0.12	0.08	1.19E-01	-0.021	0.004	**1.02E-08**	-0.036	0.007	**1.49E-07**	-0.035	0.012	4.84E-03
rs4960295	*RREB1*	a/g	0.02	0.02	4.65E-01	0.007	0.001	**1.93E-10**	0.009	0.002	**2.52E-05**	0.012	0.004	2.26E-03
rs10274998	*DGKB*	t/c	0.02	0.03	4.31E-01	0.008	0.001	**3.91E-08**	0.012	0.003	**5.87E-06**	0.011	0.005	2.09E-02
rs2157719	*CDKN2B-AS1*	c/t	-0.04	0.02	9.20E-02	-0.013	0.001	**1.30E-35**	-0.024	0.002	**5.41E-29**	-0.008	0.004	2.61E-02
rs3891783	*PLCE1*	g/c	0.04	0.02	1.01E-01	0.007	0.001	**8.01E-11**	0.011	0.002	**2.09E-07**	0.012	0.004	8.58E-04
rs1346	*SSSCA1*	t/a	-0.05	0.03	1.11E-01	-0.013	0.001	**3.88E-18**	-0.019	0.003	**5.20E-11**	-0.016	0.005	1.59E-03
rs4936099	*ADAMTS8*	c/a	-0.03	0.03	2.31E-01	-0.007	0.001	**5.16E-09**	-0.013	0.002	**3.34E-08**	-0.006	0.004	1.50E-01
13:36629905	*DCLK1*	d/r	-0.02	0.03	5.76E-01	0.007	0.001	**2.45E-08**	0.018	0.002	**1.12E-14**	-0.005	0.004	2.26E-01
rs7323428	*DCLK1*	t/g	-0.02	0.03	4.12E-01	0.007	0.001	**1.56E-08**	0.019	0.002	**8.63E-16**	-0.005	0.004	2.08E-01
rs8015152	*SIX6*	t/c	-0.06	0.02	2.03E-02	0.010	0.001	**1.34E-18**	0.024	0.002	**2.01E-26**	-0.011	0.004	5.18E-03
rs6107845	*BMP2*	a/g	0.03	0.02	2.89E-01	-0.009	0.001	**2.12E-17**	-0.017	0.002	**1.45E-15**	-0.004	0.004	3.22E-01
rs6764184	*FLNB*	t/g	0.05	0.03	5.25E-02	0.007	0.001	**1.50E-08**	0.015	0.002	**1.01E-10**	0.010	0.004	1.51E-02
rs7311936	*FAM101A*	c/g	-0.03	0.02	1.56E-01	-0.006	0.001	**1.87E-09**	-0.013	0.002	**2.35E-09**	0.003	0.004	5.21E-01
14:23388793	*RBM23*	r/d	0.02	0.03	4.00E-01	0.007	0.001	**2.13E-08**	0.013	0.003	**1.47E-07**	0.009	0.004	3.48E-02
rs3794453	*RBM23*	a/t	0.01	0.02	7.29E-01	0.007	0.001	**5.85E-08**	0.011	0.002	**2.10E-07**	0.009	0.004	2.65E-02
rs2252865	*RERE*	t/c	0.05	0.02	3.75E-02	0.005	0.001	**1.95E-05**	0.014	0.002	**6.69E-10**	0.003	0.004	5.13E-01

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs4846112	*DHRS3*	a/g	-0.02	0.03	5.23E-01	-0.005	0.001	**2.22E-04**	-0.012	0.002	**1.67E-07**	0.005	0.004	2.21E-01
rs13016883	*TRIB2*	c/g	0.02	0.03	5.48E-01	0.006	0.001	**2.53E-06**	0.016	0.002	**1.05E-11**	0.001	0.004	8.23E-01
rs35084382	*DUSP1*	c/t	-0.10	0.07	1.25E-01	-0.018	0.003	**1.56E-08**	-0.034	0.006	**1.16E-08**	-0.030	0.011	4.57E-03
rs117598310	*CRISPLD1*	t/g	-0.05	0.05	3.09E-01	0.009	0.002	**1.00E-04**	0.021	0.004	**1.32E-06**	0.022	0.008	4.96E-03
rs1360589	*CDKN2B-AS1*	c/t	-0.04	0.02	7.91E-02	-0.013	0.001	**5.05E-35**	-0.024	0.002	**4.51E-29**	-0.008	0.004	3.65E-02
rs11613189	*FAM101A*	t/c	-0.03	0.03	2.08E-01	-0.005	0.001	**4.79E-06**	-0.016	0.002	**8.66E-13**	0.002	0.004	6.49E-01
rs2251069	*DDHD1*	c/t	0.01	0.02	7.33E-01	-0.006	0.001	**7.12E-08**	-0.013	0.002	**5.69E-10**	0.001	0.004	7.33E-01
rs6598351	*FAM169B*	t/c	-0.02	0.03	5.30E-01	0.006	0.001	**2.34E-05**	0.012	0.003	**1.19E-05**	-0.004	0.005	3.83E-01
rs11646917	*SALL1*	t/g	-0.01	0.03	6.71E-01	-0.009	0.001	**3.43E-10**	-0.015	0.003	**2.54E-09**	-0.015	0.005	1.16E-03
rs11867840	*BCAS3*	g/a	0.04	0.03	1.04E-01	-0.006	0.001	**3.89E-06**	-0.018	0.002	**1.15E-13**	0.012	0.004	7.36E-03
rs6054375	*BMP2*	t/g	0.03	0.03	2.57E-01	-0.010	0.001	**4.10E-15**	-0.018	0.002	**8.77E-16**	-0.003	0.004	4.72E-01
rs3791679	*EFEMP1/PNPT1*	g/a	0.04	0.03	1.66E-01	-0.005	0.001	**1.02E-04**	-0.013	0.002	**2.94E-08**	0.003	0.004	4.93E-01
rs12494328	*FLNB*	a/g	0.04	0.03	1.56E-01	0.006	0.001	**1.43E-06**	0.016	0.002	**4.39E-11**	0.009	0.004	3.96E-02
6:36592986	*CDKN1A*	d/r	-0.02	0.03	5.08E-01	0.006	0.001	**1.60E-05**	0.015	0.003	**7.85E-09**	-0.006	0.005	2.04E-01
rs72852338	*CDKN1A*	c/a	-0.02	0.03	5.19E-01	0.006	0.001	**2.74E-05**	0.014	0.003	**2.26E-08**	-0.005	0.005	2.96E-01
rs1074407	*TRIOBP*	t/a	0.11	0.02	**3.42E-06**	0.006	0.001	**2.66E-07**	0.012	0.002	**1.17E-08**	0.008	0.004	3.15E-02

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with vertical cup-disc ratio that are Bonferroni significantly associated with cup area are shown in bold (p-value < 5.31×10^{-4}; 0.05/94).Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Table2c. Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with vertical cup-disc ratio and show an association with disc area

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
rs1192414	*CDC7/TGFBR3*	a/g	0.06	0.03	5.39E-02	0.014	0.001	**8.18E-24**	0.007	0.003	1.07E-02	0.087	0.005	**3.78E-74**
rs10753787	*F5*	t/c	-0.03	0.02	1.80E-01	-0.007	0.001	**2.11E-09**	-0.005	0.002	1.84E-02	-0.019	0.004	**9.12E-07**
rs2920293	*PSCA*	g/c	0.00	0.02	8.67E-01	-0.006	0.001	**4.95E-09**	-0.007	0.002	7.60E-04	-0.015	0.004	**5.41E-05**
rs4658101	*CDC7/TGFBR3*	a/g	0.06	0.03	4.24E-02	0.013	0.001	**2.35E-23**	0.007	0.003	1.09E-02	0.089	0.005	**2.35E-82**
1:169530520	*F5/SELP*	i/r	0.02	0.03	4.44E-01	0.007	0.001	**6.29E-07**	0.005	0.003	5.33E-02	0.032	0.005	**5.13E-13**
rs2239854	*F5/SELP*	a/g	0.03	0.03	2.73E-01	0.006	0.001	**6.94E-07**	0.005	0.002	4.94E-02	0.030	0.004	**3.43E-13**
rs9843102	*ABI3BP*	a/g	0.00	0.03	9.91E-01	-0.006	0.002	**2.01E-04**	-0.002	0.003	5.89E-01	-0.036	0.005	**3.99E-12**
8:88744441	*DCAF4L2*	d/r	-0.01	0.02	6.98E-01	0.006	0.001	**4.50E-07**	0.006	0.002	4.47E-03	0.026	0.004	**7.15E-12**
rs6468996	*DCAF4L2*	t/c	0.00	0.02	9.19E-01	0.005	0.001	**1.86E-07**	0.006	0.002	2.11E-03	0.025	0.004	**8.07E-12**
rs61101201	*ELP4/PAX6*	g/t	0.02	0.03	5.33E-01	0.006	0.001	**1.85E-06**	0.005	0.002	3.91E-02	0.028	0.004	**3.52E-11**
rs56385951	*CARD10*	a/g	-0.06	0.04	8.65E-02	0.011	0.002	**1.36E-11**	0.008	0.003	8.50E-03	0.047	0.006	**2.48E-17**
1:3046430	*PRDM16*	i/r	-0.04	0.04	4.19E-01	0.007	0.002	**5.00E-04**	-0.001	0.004	7.18E-01	0.044	0.007	**5.15E-10**
rs12028027	*PRDM16*	c/t	-0.03	0.04	5.03E-01	0.007	0.002	**2.02E-04**	-0.001	0.004	8.62E-01	0.043	0.007	**6.02E-10**

For these SNPs, the associations with the other traits are also included. Here the SNPs genome-wide significantly associated with vertical cup-disc ratio that are Bonferroni significantly associated with disc area are shown in bold (p-value < 5.31×10^{-4}; 0.05/94).Nearest gene, reference NCBI build37; β, effect size on effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Table 3 Single nucleotide polymorphisms (SNPs) that are genome-wide significantly associated with optic nerve head parameters (cup area and disc area)

			IOP			VCDR			Cup area			Disc area		
SNP	**Nearest gene**	**A1/A2**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**B**	**SE**	**P**
1:227562773	*CDC42BPA*	d/r	-0.10	0.05	2.74E-02	0.003	0.002	2.39E-01	0.024	0.004	**4.60E-09**	-0.055	0.007	**4.93E-14**
rs73102394	*CDC42BPA*	t/c	-0.09	0.04	3.92E-02	0.003	0.002	1.60E-01	0.022	0.004	**2.53E-08**	-0.053	0.007	**6.79E-14**
rs11811982	*CDC42BPA*	a/c	-0.12	0.05	1.21E-02	0.004	0.002	5.48E-02	0.027	0.004	**1.32E-10**	-0.062	0.008	**1.74E-16**
rs10021731	*UGT8*	c/t	0.01	0.02	8.26E-01	-0.002	0.001	5.60E-02	-0.002	0.002	2.63E-01	-0.020	0.004	**2.44E-07**
rs12220165	*CTNNA3*	g/c	0.02	0.03	5.94E-01	-0.004	0.002	1.41E-02	-0.004	0.003	1.76E-01	-0.023	0.005	**1.75E-05**
rs787541	*U6, GADD45A*	c/g	0.07	0.03	7.17E-03	0.002	0.001	7.26E-02	0.002	0.002	5.06E-01	0.023	0.004	**3.16E-08**
rs1367187	*DIRC3*	c/t	-0.07	0.03	9.01E-03	0.002	0.001	2.63E-01	-0.002	0.003	4.79E-01	0.026	0.005	**5.96E-09**
rs2443724	*VGLL4*	c/g	0.00	0.02	8.77E-01	-0.003	0.001	1.50E-02	0.000	0.002	9.47E-01	-0.022	0.004	**1.40E-08**
rs1013830	*CTNNA3*	t/c	0.00	0.05	9.53E-01	-0.007	0.002	4.70E-03	-0.004	0.005	3.96E-01	-0.045	0.008	**2.00E-08**

For these SNPs, the associations with the other traits are also included. SNPs that are Bonferroni significantly associated with other traits are shown in bold (p-value < 5.31×10^{-4}; 0.05/94). In the first rows, the SNPs genome-wide significantly associated with cup area are shown. Next, SNPs associated with only disc area, are shown. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; β, effect size on the endophenotype (IOP, VCDR, cup area or disc area) based on allele A1; SE, standard error of the effect size; i, insertion; d, deletion; r, reference.

Figure 1. Overlap between the genes associated with one or more endophenotypes. Genes with genome-wide significant association for at least one trait are shown. These genes are counted as overlapping genes if they are Bonferroni significantly associated with the other trait(s). Chr 11p11.2 (see intraocular pressure circle) means a region on chromosome 11p11.2 that is associated with IOP and has many genes in it; the likely causative gene in this region is not identified yet. Genes in bold are genes associated with primary open-angle glaucoma (POAG) in our meta-analysis of four case-control studies.*Genes associated with familial forms of POAG (e.g. *MYOC* and *OPTN*) or found in case-control association studies which did not show an association with the endophenotypes explored in this study.

To further characterize the overlap in biological functions, gene set enrichment of loci associated with IOP and optic disc parameters was performed using DEPICT[19]. We first investigated enriched pathways or gene sets using only genome-wide associated SNPs. No significant pathways were found after FDR correction. However, pathways involved in metabolic processes such as "increased circulating leptin level", "abnormal fat cell morphology" and "increased insulin sensitivity" were suggestive when we analyzed the list of SNPs associated with VCDR, cup area and disc area (FDR<0.2, see **S11 Table**). We next searched for enriched pathways using suggestive SNPs (p-value $<1.0 \times 10^{-5}$). We further investigated potential overlap in pathways across the endophenotypes, and found 57 significant pathways when using VCDR, cup area and IOP variants; and 100 pathways when analysing suggestive VCDR, cup area and disc area variants. Note that in the first analysis we investigated pathways enriched when IOP genes are taken into account, while in the second one we analysed genes influencing the optic nerve head characteristics. Due to a high degree of redundancy between pathways, we clustered the significant pathways into meta-pathways, resulting in 11 meta-pathways for VCDR, cup area and IOP (**Figure 2a**, **S12 Table**); and 17 for VCDR, cup area, and disc area (**Figure 2b**, **S13 Table**). Most of the gene sets found in both analyses highlighted pathways involved in cell differentiation, notch signaling, regulatory DNA binding and embryonic development, which reflects the pathways found when VCDR and CA variants are analyzed (**S17 Fig**). Furthermore, we found "abnormal fat cell morphology" and "abnormal liver morphology" significantly enriched; a key gene in these pathways is *ABCA1*. When IOP genes are included the elongation factor, RNA Polymerase II (ELL2) protein complex" shows an enrichment. When disc area genes are included, pathways such as "blood vessel development", "protein import into nucleus", "Thrombospondin 1 (THBS1) and SMAD3 protein complex", and "abnormal eye morphology" were significant. Key genes in the latter include: *CDKN2B, FAT4, LRIG3, SIX6, COL8A1, SOX11, RND3, BOC, WNT2B* and *CYP26A1.*

Figure 2. Pathways significantly enriched for: **A**) Loci associated with vertical cup-disc ratio, cup area and intraocular pressure (p-value $<7.0 \times 10^{-6}$ in the GWAS). In total 11 meta-pathways were identified after clustering the 57 pathways identified by DEPICT. **B**) Loci associated with vertical cup-disc ratio,

cup area and disc area (p-value <1.0 x 10^{-5}). In total 17 meta-pathways were identified after clustering the 100 pathways identified by DEPICT. In both figures meta-pathways are represented by nodes coloured according to statistical significance, and edges are scaled according to the correlation between meta-pathways. *The pathway "Abnormal eye morphology" clustered with the meta-pathway "Chordate embryonic development". ELL2=Elongation Factor, RNA Polymerase II, , DVL3= Dishevelled Segment Polarity Protein 3, THBS1=Thrombospondin 1, RFX2= Regulatory Factor X, 2. MDFI=MyoD Family Inhibitor.

From endophenotypes to primary open-angle glaucoma

Of the 75 independent (i.e. $R^2 < 0.8$) SNPs that were associated with one or more of the endophenotypes, 32 were nominal significantly associated with POAG in a meta-analysis of 6,429 cases and 41,404 controls (p-value <0.05; the chance that 32 SNPs of 75 SNPs have a p-value <0.05 is < 2.2 x 10^{-16}), and 11 independent SNPs were Bonferroni significantly associated with POAG (p-value 0.05/75 = 6.67 x 10^{-4}) (**Table 4**). Of these, the rs2487048 in the *ABCA1* gene and the 11:120357425 in the *ARHGEF12* showed high heterogeneity (I^2). To estimate the common effect size we performed a random effect meta-analysis. The odds ratio (OR) remained almost the same for both variants, although p-values were not significant after adjusting for multiple testing, which is in line with the heterogeneity observed. All other nine SNPs surpassed the Bonferroni threshold for significance in both fixed and random-effect models. The association between *CDKN1A* and POAG is novel (OR = 1.14, p-value = 7.4 x 10^{-7}). In our previous paper, the SNP rs6054374 near to *BMP2* was already associated with POAG (OR = 0.92, p-value 3.74 x 10^{-3}), but the most significantly associated SNP in the current meta-analysis rs6107845 near to *BMP2* shows a slightly larger effect on POAG (OR = 0.89, p-value = 8.52 x 10^{-6}). *CDKN1A* is a novel gene in the same gene family as *CDKN2B*, a gene previously associated to glaucoma. Both *CDKN1A* and *CDKN2B* are cell-cycle genes.

Table 4. **Association with primary open-angle glaucoma in a meta-analysis of four independent glaucoma case-control studies (ANZRAG, NEIGHBORHOOD, Singapore, and Southampton).**

	Nearest gene	A1/A2	OR	OR (R)	95% CI	P-value	P-value (R)	Direction	I2	P-value of heterogeneity
IOP SNPs										
rs10918274	*TMCO1*	t/c	1.39	1.39	1.3-1.5	**2.75E-19**	**1.37E-09**	++++	38.4	1.82E-01
rs7635832	*FNDC3B*	g/t	0.89	0.91	0.83-0.95	1.41E-03	3.65E-02	---?	33.9	2.20E-01
rs10281637	*CAV1/CAV2*	c/t	1.13	1.13	1.07-1.20	**2.32E-05**	**2.32E-05**	++++	0	4.89E-01
rs2487048	*ABCA1*	a/g	1.26	1.26	1.19-1.33	**2.65E-15**	3.82E-03	++++	82.9	5.53E-04
rs8176741	*ABO1*	a/g	1.07	1.04	0.99-1.17	7.36E-02	5.25E-01	-+-+	58.5	6.51E-02
rs7944735	Many genes *(NUP160, PTPRJ)*	c/g	1.06	1.07	1.01-1.13	2.99E-02	2.99E-02	++++	0	8.99E-01
11:120357425	*ARHGEF12*	d/r	1.16	1.19	1.09-1.23	**1.52E-06**	3.02E-02	++++	83.2	4.65E-04
rs55796939	*ADAMTS8*	t/c	1.07	1.17	0.94-1.24	2.72E-01	4.46E-01	+?--	78.6	9.35E-03
rs9913911	*GAS7*	g/a	0.80	0.80	0.76-0.84	**1.08E-17**	**1.08E-17**	----	0	7.50E-01
VCDR SNPs										
rs1925953	*RPE65*	t/a	1.07	1.10	1.02-1.13	4.21E-03	2.01E-02	++++	46.7	1.31E-01
rs1192414	*CDC7/TGFBR3*	a/g	1.08	1.08	1.02-1.16	9.26E-03	9.26E-03	++++	0	7.27E-01
rs10753787	*F5*	t/c	0.97	0.97	0.93-1.03	3.67E-01	3.67E-01	----	0	9.92E-01
rs6804624	*COL8A1*	c/t	0.99	0.99	0.94-1.05	8.14E-01	8.14E-01	---+	0	8.42E-01
rs72759609	*PDZD2*	c/t	0.90	0.91	0.83-0.99	3.20E-02	3.20E-02	----	0	9.53E-01
rs114503346	*DUSP1*	t/c	1.00	1.00	0.80-1.25	9.99E-01	8.80E-01	+?-+	42	1.78E-01
rs4960295	*RREB1*	a/g	0.99	1.00	0.95-1.05	9.50E-01	9.09E-01	-+-+	4.6	3.70E-01
rs10274998	*DGKB*	t/c	1.03	1.04	0.98-1.10	2.16E-01	2.16E-01	++-+	0	5.38E-01
rs2157719	*CDKN2B-AS1*	c/t	0.69	0.69	0.66-0.74	**1.29E-40**	**1.29E-40**	----	0	5.67E-01
rs1900005	*ATOH7*	a/c	1.01	1.01	0.96-1.07	6.98E-01	6.77E-01	+-++	5.1	3.67E-01
10:96008348	*PLCE1*	d/r	1.02	1.04	0.97-1.09	3.38E-01	3.15E-01	+-+?	35.3	2.13E-01
rs1346	*SSSCA1*	t/a	0.90	0.91	0.85-0.97	2.41E-03	2.41E-03	----	0	9.04E-01
rs4936099	*ADAMTS8*	c/a	0.94	0.94	0.9-1.00	5.75E-02	5.75E-02	----	0	9.63E-01
rs324780	*TMTC2*	g/a	0.93	0.93	0.89-0.99	1.35E-02	1.35E-02	----	0	7.69E-01
13:36629905	*DCLK1*	d/r	0.99	0.99	0.94-1.05	7.53E-01	8.00E-01	--+-	6.2	3.62E-01
rs8015152	*SIX6*	t/c	1.21	1.19	1.16-1.28	**3.90E-15**	**7.08E-05**	++++	62.4	4.62E-02
rs4299136	*ASB7*	c/g	1.03	1.03	0.97-1.10	3.55E-01	3.55E-01	++-+	0	8.29E-01
16:51461915	*SALL1*	i/r	0.94	0.94	0.89-1	3.85E-02	3.85E-02	----	0	7.82E-01
rs6107845	*BMP2*	a/g	0.89	0.91	0.85-0.94	1.02E-05	6.94E-03	----	43.1	1.53E-01
rs5752773	*CHEK2*	g/c	0.92	0.92	0.88-0.98	4.63E-03	4.63E-03	----	0	9.12E-01
rs2092172	*CARD10*	a/g	0.97	0.98	0.92-1.04	4.35E-01	4.35E-01	--+-	0	7.76E-01
rs6764184	*FLNB*	t/g	1.07	1.02	1.02-1.13	5.73E-03	7.66E-01	++-+	86.1	8.14E-05
rs7717697	*VCAN*	c/t	0.98	0.98	0.93-1.04	5.26E-01	5.26E-01	---?	0	7.30E-01
rs2920293	*PSCA*	g/c	1.03	1.03	0.98-1.09	2.25E-01	2.25E-01	++-?	0	3.79E-01
rs1681739	*ENO4*	t/c	1.02	1.03	0.97-1.08	3.92E-01	3.99E-01	+--+	49.2	1.16E-01
rs7311936	*FAM101A*	c/g	0.99	1.00	0.95-1.04	8.12E-01	8.59E-01	+---	11	3.38E-01
14:23388793	*RBM23*	r/d	1.03	1.03	0.98-1.1	1.83E-01	1.83E-01	+++?	0	4.61E-01
Cup area SNPs										
rs2252865	*RERE*	t/c	1.11	1.11	1.06-1.18	5.76E-05	2.87E-02	++-+	59.3	6.10E-02
rs4846112	*DHRS3*	a/g	0.95	0.96	0.91-1.01	1.18E-01	1.18E-01	----	0	5.53E-01
1:227562773	*CDC42BPA*	d/r	0.87	0.90	0.79-0.97	1.14E-02	2.11E-01	--+?	48.6	1.43E-01
rs13016883	*TRIB2*	c/g	1.08	1.08	1.03-1.14	4.25E-03	4.25E-03	+++?	0	8.63E-01
rs35084382	*DUSP1*	c/t	1.04	1.05	0.85-1.29	6.72E-01	6.72E-01	+?-+	0	3.91E-01
rs117598310	*CRISPLD1*	t/g	1.08	1.09	1-1.19	5.39E-02	5.39E-02	+++	0	8.01E-01
rs1360589	*CDKN2B-AS1*	c/t	0.69	0.69	0.66-0.73	**1.90E-42**	**1.90E-42**	----	0	6.47E-01
rs10998036	*ATOH7*	c/g	1.01	1.02	0.96-1.08	5.42E-01	5.72E-01	+---	26	2.55E-01
10:96008348	*PLCE1*	d/r	1.02	1.04	0.97-1.09	3.38E-01	3.15E-01	+-+?	35.3	2.13E-01
rs1346	*SSSCA1*	t/a	0.90	0.91	0.85-0.97	2.41E-03	2.41E-03	----	0	9.04E-01
rs482507	*TMTC2*	c/t	0.94	0.94	0.89-0.99	2.03E-02	2.03E-02	----	0	7.46E-01
rs11613189	*FAM101A*	t/c	0.99	0.99	0.95-1.05	8.25E-01	7.77E-01	++--	18.5	2.98E-01
rs7323428	*DCLK1*	t/g	0.99	1.00	0.94-1.05	7.83E-01	8.87E-01	+-+-	13.6	3.25E-01
rs2251069	*DDHD1*	c/t	0.95	0.96	0.91-1.00	7.62E-02	7.62E-02	--+-	0	4.08E-01
rs4436712	*SIX6*	t/g	1.24	1.23	1.19-1.31	**5.77E-18**	**1.52E-07**	++++	48.8	1.19E-01

	Nearest gene	A1/A2	OR	OR (R)	95% CI	P-value	P-value (R)	Direction	I2	P-value of heterogeneity
Cup area SNPs										
rs6598351	*FAM169B*	t/c	0.99	0.99	0.93-1.06	8.06E-01	8.06E-01	-+--	0	7.11E-01
rs11646917	*SALL1*	t/3g	0.98	0.98	0.93-1.04	5.49E-01	5.49E-01	--++	0	5.97E-01
rs11867840	*BCAS3*	g/a	1.06	1.06	1.01-1.13	1.83E-02	2.12E-02	++++	8.3	3.51E-01
rs6054375	*BMP2*	t/g	0.89	0.91	0.85-0.94	8.52E-06	9.93E-03	----	47.1	1.29E-01
rs738722	*CHEK2*	t/c	0.93	0.93	0.89-0.99	1.26E-02	1.26E-02	----	0	9.05E-01
rs3791679	*EFEMP1/PNPT1*	a/g	0.96	0.96	0.92-1.02	2.23E-01	2.23E-01	----	0	5.51E-01
rs12494328	*FLNB*	a/g	1.13	1.13	1.07-1.2	**1.28E-05**	**5.89E-04**	++-+	26.9	2.50E-01
rs6804624	*COL8A1*	c/t	0.99	0.99	0.94-1.05	8.14E-01	8.14E-01	---+	0	8.42E-01
6:36592986	*CDKN1A*	d/r	1.14	1.15	1.09-1.21	**7.74E-07**	**1.04E-04**	++++	36.6	1.93E-01
rs2684249	*HSF2*	c/t	0.92	0.94	0.88-0.97	1.08E-03	1.66E-01	---+	63.3	4.25E-02
rs8176672	*ABO*	t/c	1.00	1.00	91-1.11	9.49E-01	9.49E-01	-+-?	0	3.69E-01
rs4936099	*ADAMTS8*	c/a	0.94	0.94	0.9-1.00	5.75E-02	5.75E-02	----	0	9.63E-01
rs34222435	*ASB7*	t/c	1.03	1.03	0.97-1.10	3.66E-01	3.66E-01	++-+	0	8.74E-01
rs1074407	*TRIOBP*	t/a	1.04	1.04	1.00-1.10	4.92E-02	8.66E-02	++++	32.9	2.15E-01
Disc Area SNPs										
rs4658101	*CDC7/TGFBR3*	a/g	1.08	1.08	1.02-1.16	7.81E-03	7.81E-03	++++	0	7.22E-01
1:169530520	*F5/SELP*	i/r	1.01	1.02	0.96-1.08	5.40E-01	5.40E-01	++-?	0	7.14E-01
rs11811982	*CDC42BPA*	a/c	0.87	0.90	0.8-0.97	1.19E-02	8.28E-02	--++	20.5	2.87E-01
rs9843102	*ABI3BP*	a/g	0.92	0.92	0.86-0.98	1.37E-02	1.37E-02	----	0	6.24E-01
rs10021731	*UGT8*	c/t	1.01	1.01	0.96-1.06	6.82E-01	6.82E-01	--++	0	6.50E-01
8:88744441	*DCAF4L2*	d/r	1.03	1.04	0.99-1.09	0.1225	1.39E-01	++-+	4.9	3.68E-01
rs12220165	*CTNNA3*	g/c	1.08	1.09	1.01-1.16	1.14E-02	1.14E-02	++++	0	9.04E-01
rs7916410	*ATOH7*	t/c	1.00	1.00	0.96-1.06	7.63E-01	7.45E-01	+-++	3.9	3.73E-01
rs61101201	*ELP4/PAX6*	g/t	1.00	1.00	0.94-1.06	9.77E-01	9.77E-01	-+-?	0	9.63E-01
rs442376	*TMTC2*	c/t	1.04	1.05	0.99-1.10	7.94E-02	7.94E-02	-+++	0	6.82E-01
rs1345467	*SALL1*	g/a	1.07	1.07	1.01-1.14	1.86E-02	1.86E-02	++++	0	8.73E-01
rs5762752	*CHEK2*	c/g	0.92	0.92	0.88-0.98	4.90E-03	4.90E-03	----	0	8.29E-01
rs56385951	*CARD10*	a/g	0.99	1.00	0.92-1.07	9.15E-01	9.15E-01	+-+-	0	9.88E-01
1:3046430	*PRDM16*	i/r	0.97	0.98	0.87-1.10	7.13E-01	8.72E-01	+--?	63.9	6.28E-02
rs787541	*U6, GADD45A*	c/g	0.98	0.98	0.94-1.04	6.10E-01	9.06E-01	--++	50.7	1.08E-01
rs1367187	*DIRC3*	c/t	0.95	0.96	0.9-1.01	1.11E-01	4.12E-01	+-+-	46.1	1.35E-01
rs2443724	*VGLL4*	c/g	0.91	0.91	0.87-0.97	1.04E-03	2.61E-02	--+-	38	1.84E-01
rs11129176	*RARB*	a/g	0.99	1.00	0.94-1.05	8.85E-01	9.93E-01	+---	40.4	1.69E-01
rs1997404	*COL8A1*	g/t	1.00	1.00	0.95-1.06	9.60E-01	9.60E-01	-+++	0	6.18E-01
rs34935520	*SIX6*	g/a	1.26	1.26	1.20-1.33	**2.82E-20**	**6.73E-14**	++++	21.5	2.81E-01
rs60779155	*ASB7*	a/g	1.02	1.03	0.96-1.10	4.52E-01	4.52E-01	+--+	0	5.02E-01

Results are shown for the most significantly associated single nucleotide polymorphisms from the endophenotype analyses. Nearest gene, reference NCBI build37; A1, reference allele; A2, other allele; OR, estimated odds ratio for allele A1; OR (R), estimated odds ratio for allele A1 in random effect meta-analysis; 95% CI, confidence interval; P-value (R), p-value in random effect meta-analysis; I^2 statistic measuring heterogeneity on a scale of 0% to 100%; i, insertion; d, deletion; r, reference.

Expression of cdkn1a after knockdown of six6b in zebrafish

In a previous zebrafish model[20], we demonstrated that downregulation of the POAG gene *six6b* leads to up-regulation of *CDKN2B*, another well-known POAG gene. This zebrafish model, rather than

a POAG model, allowed us to investigate the downstream effects of *six6b* depletion, and possible mechanisms underlying POAG. In this study, we tested whether knockdown of *six6b* also alters the expression of the newly identified *cdkn1a* gene *in vivo*. Knockdown of *six6b* was achieved using morpholino technology[20]. 85% of the knockdown embryos showed a small eye phenotype, reduced optic nerve thickness and an up-regulation of the expression levels of *cdkn2a/cdkn2b*, as observed in previous studies (n=220)[20, 21]. In zebrafish, there is only one gene which is analogous to the human *CDKN2A* and *CDKN2B* and it is referred to in this paper as *cdkn2a/cdkn2b*. Both *SIX6*and *CDKN2B* are part of the abnormal eye morphology pathway found with DEPICT, which is in line with previous findings. *In silico* analyses showed that *SIX6* binds to both *CDKN2B* (core score = 1) and *CDKN1A* (core score = 0.812). . We evaluated the expression levels of *cdkn1a* in *six6b* deficient embryos by RT-qPCR. A 41-fold overexpression of *cdkn1a* in the eye of *six6b* knockdown embryos was found (p-value = 0.001) (**Figure 3**), showing that *in vivo* downregulation of *six6b* affects the expression levels not only of *cdkn2a/cdkn2b* but also of *cdkn1a*, likely by binding to their sequence, repressing their expression.

Figure 3. ***cdkn1a*** **mRNA expression change**

Overexpression of *cdkn1a* and *cdkn2a/cdkn2b* in response to *six6b* depletion is shown. All samples expression were normalized to the control gene *sdha*. Relative expression was calculated by setting the wild-type expression level at 1. Values represent mean ± standard error of the mean. *P<0.05; **P<0.005.

Discussion

This meta-analysis within the IGGC identified a novel genomic region associated with IOP, nine genomic regions associated with VCDR, five with cup area, and six with disc area. Eleven genomic regions were associated with POAG. Of these regions, the association between *CDKN1A* and POAG is novel.

We identify some specific loci that underlie the genetic correlation between IOP and VCDR described earlier[12]. *ADAMTS8* and *ABO* were genome-wide significant for both IOP and VCDR or cup area. Variants found close to *ABO* (rs8176672 for cup area and rs8176741 for IOP) are in LD (r2 >0.85) with rs12216891, which lies in an enhancer and promoter histone mark, suggesting a potential regulatory mechanism in that region. Furthermore, *TRIOBP* is genome-wide significant for cup area, and reached a p-value of 3.42×10^{-6} for IOP. Interestingly, *TRIOBP* is approximately 180 kb away from *CARD10* which is associated with disc area. There is a large overlap between VCDR/cup area and disc area. Since VCDR is related to disc area, it might be that the effect found for VCDR is due to the effect of disc area. Most of these overlapping genes are still Bonferroni significant in the cup area analysis in which we corrected for disc area. Only *CDC7/TGFBR3* and *F5* are genome-wide significant for VCDR as well as for disc area, but the effect is negligible after correction for disc area, suggesting that these two genes play primarily a role in disc area.

When suggestive SNPs (p-value $<1.0 \times 10^{-5}$) for VCDR and cup area are analyzed together using DEPICT, we found an enrichment of pathways involved in cell differentiation, development, regulatory DNA binding and Notch signaling. Including disc area SNPs to the VCDR and cup area analysis reveals additional joint pathways: 1) eye and blood vessel development, 2) cancer, 3) protein import into nucleus, and 4) thrombospondin 1 and SMAD3 complexes, related to the extracellular matrix. Of interest, known POAG genes also fit in these pathways identified in this paper based on

endophenotypes: *GAS7* and *SIX6* play a role during development[20, 22], *TGFBR3* has been implicated in extracellular matrix regulation[23] and in cancer as well as *GMDS*[24].

The extracellular matrix pathway has been previously implicated in optic nerve degeneration[14], and emerges in the DEPICT analyses. Both *ADAMTS8* and *COL8A1* have a role in this pathway. The encoded protein of the novel identified gene *VCAN* (versican) is also a major component of the extracellular matrix. Another member of the ADAMTS family (*ADAMTS5*) plays a role in the regulation of versican[25]. Interestingly, mutations in *VCAN* have been implicated in several ophthalmologic disorders[26].

The gene *CDKN2B* encodes a cyclin-dependent kinase inhibitor. Its antisense (*CDKN2B-AS1)* was one of the first POAG genes identified by GWAS. The gene *CDKN1A*, also known as *p21*, *CIP-1* or *WAF-1*, is a gene from the same family as *CDKN2B* and also encodes a cyclin-dependent kinase inhibitor. Upregulation of *CDKN1A* causes G1 arrest and inhibits proliferation of the cell. Herein, for the first time, we provide genome-wide significant evidence for association of *CDKN1A* variants with cup area. Two prior small cohort studies suggested a possible role of *CDKN1A* in POAG. Tsai et al.[27] found an association between a codon 31 polymorphism in *CDKN1A* and POAG in 58 patients and 59 controls from China (OR = 2.39 [1.14-5.01]). Saglar et al. found no statistically significant association between the codon 31 polymorphism and POAG in 75 patients and 119 controls from Turkey (OR = 1.70, p-value = 0.25)[28]. Our study provides strong evidence for the role of *CDKN1A* in POAG risk in a large sample consisting of 6,429 cases and 41,404 controls and shows the first convincing evidence for association of *CDKN1A* and POAG in individuals of European descent. In addition, our *in vivo* studies in embryonic zebrafish eye showed that knockdown of *six6b* upregulates both *cdkn2a/cdkn2b* and *cdkn1a*. In a recent study, Skowronska-Krawczyk et al. showed that *SIX6* regulates the expression of *CDKN2A*, located in the same locus of *CDKN2B* and *CDKN2B-AS1*. The authors proposed that IOP elevation leads to *CDKN2A* upregulation through increased expression of *SIX6*. Increased *CDKN2A* causes senescence of retinal ganglion cells[29]. In our study, we found that

depletion, instead of upregulation of *six6b* leads to increased levels of *cdkn2a/cdkn2b*. Dual transcription activities of the SIX proteins have been described before. During development, for example, depending on the developmental context, SIX proteins can activate or repress their target genes[30]. Interestingly, the outcome of either *SIX6* as transcriptional repressor or activator seems to be the upregulation of the CDK inhibitor genes (*CDKN1A, CDKN2A* and *CDKN2B*). More comprehensive studies at the individual tissue level e.g. retinal ganglion cell layer or optic nerve should be performed to evaluate the molecular mechanisms behind the upregulation of the CDK inhibitor genes in the context of normal and high tension glaucoma.

The synthesis of *CDKN1A* is increased by the binding of p53 to p53-specific DNA consensus sequence[31, 32]. It has been suggested that p53 plays a role in POAG, especially in POAG with paracentral visual field loss[33].In a *p53* knockout mouse model, less apoptosis was observed after induction of high IOP. Suggesting that the downregulation of *p53* could attenuate the cell damage caused by high IOP levels[29]. Other genes also play a role in p53. *GADD45A* is involved in growth arrest through p53 dependent and independent mechanisms[31, 34] and can interact via *CDKN1A*[35]. Other novel identified genes might also play a role in p53-induced apoptosis. It has been shown that the secreted *pdzd2* protein activates p53 by transcriptional regulation[36]. Also *RREB1* has an effect on p53 by binding to its promotor and transactivates its expression[37]. This gene encodes a zinc finger transcription factor. This can bind to the RAS-responsive element of the calcitonin gene promotor which subsequently increases the expression of calcitonin. Calcitonin may be involved in the Ras/Raf signaling cascade that plays a role in the morphogenesis of retinal ganglion cells, the cell type affected by glaucoma, during neurogenesis[38]. Also *PSCA* is probably involved in p53-related pathways[39]. Other genes play a role in apoptosis or cell growth via other pathways than p53: *VGLL4* inhibits Bax- and TNFa-induced apoptosis[40] and *DGKB* is a regulator of diacylglycerol, which is important for cell growth and differentiation. *UGT8* plays a role in the

biosynthesis of the sphingolipids of myelin membranes of the central and peripheral nervous system; sphingolipids are also implicated in apoptosis[41].

Another interesting novel gene is *RPE65* (retinal pigment epithelium -specific protein 65kDa). This gene has been associated with retinitis pigmentosa [42, 43] and Leber congenital amaurosis type 2 (LCA2)[44]. As the name implies, the encoded protein is located in the retinal pigment epithelium[45]. It is involved in the conversion of all-trans retinal to 11-cis retinal, which is a necessary step in the visual cycle. Both diseases (RP and LCA2) are not characterized by an excavation of the optic nerve head. However, we have checked several online databases for expression in different tissues. In the eye, it is also highly expressed in the optic nerve head (**S8 and S9 Tables**) suggesting that this gene could be involved in other ocular processes . Little expression is found in the brain, with no expression in other tissues or organs in the body. Future studies are necessary to confirm our finding.

Of the genes identified by gene-based testing, *C9* (complement component 9) is especially interesting. Its protein is part of the membrane attack complex (MAC), together with the proteins C5b, C6, C7, and C8. This complex activates several steps that lead to cell death, and cells protect themselves by removing the complex through endocytosis. Caveolin is one of the proteins involved in endocytosis and the *CAV1/CAV2* genes are associated with IOP and POAG. It has been shown that inhibition of caveolin-1 inhibits the endocytosis of MAC[46].

To our best knowledge, this meta-analysis is the largest study of IOP and optic nerve head parameters to date, using well-characterized datasets from populations world-wide. A limitation of our study is the lack of an available dataset for replication of the novel associations detected by combined European and Asian ancestry samples. However, the heterogeneity of these novel genomic regions is generally low in the meta-analysis. For VCDR, cup area, and disc area we have identified novel SNPs in the analysis of individuals with European ancestry. Of the nine novel associations found

in these populations (*RPE65*, *PDZD2*, *RREB1*, *DGKB* for VCDR; *CDC42BPA, CRISPLD1* and *FAM169B* for cup area; and *CTNNA3* and *UGT8* for disc area), only *RREB1* was nominally significant in the individuals with Asian ancestry. Five of the seven non-significant SNPs in the individuals with Asian ancestry had an effect estimate in the same direction. As the analysis in individuals with Asian ancestry contains a smaller number of individuals, this could be due to lack of power.

We have identified 21 genetic variants associated with POAG endophenotypes. These association results do not imply that the variants described here have a causal effect. Fine-mapping and functional studies are required to identify the causal variants tagged by our findings and the exact molecular mechanisms involved in POAG. In conclusion, we have found novel genomic regions associated with the POAG endophenotypes: IOP, VCDR, cup area, and disc area. Although the overlap between IOP-loci and the optic disc parameters-loci is not large , this is the first study showing a genome-wide significant evidence of the genetic correlation between IOP and VCDR; we expect that larger sample sizes and improved imputation accuracy may help to find more of the loci underlying the genetic correlation between these two endophenotypes. Of the novel associations, *CDKN1A* is strongly associated with POAG, This finding is in line with other studies[29], pointing to the CDK-inhibitor genes as key players in the development of POAG. The p53 pathway has been implicated in POAG, intriguingly, p53 has been also related to the CDK-inhibitors and to four of the new genes pointed out by this study (*GADD45A*, *PDZD2*, *RREB1* and *PSCA*). Functional studies need to be performed to assess the role of *p53* and CDK-inhibitors in the pathophysiology of POAG. A more comprehensive study of these mechanisms may inform the development of new therapies for POAG.

Methods

Study design

We performed a meta-analysis on directly genotyped and imputed SNPs to the 1000 Genomes reference panel. We analyzed four outcomes: IOP, VCDR, cup area, and disc area. In the first stage, we included 22,489-29,578 individuals with European ancestry. Subsequently, we evaluated the genome-wide significant SNPs from the first stage in 7,307-8,373 individuals with Asian ancestry. Finally, we performed a meta-analysis of GWAS summary findings from all individual studies including individuals with European and Asian ancestry. We subsequently tested the effect of all genome-wide significant SNPs on POAG in four independent case-control studies.

Subjects, phenotyping and genotyping

All 19 studies included in this meta-analysis are part of the IGGC (**S1a Table).**Details for each individual study can be found in **S1b**, **S1c,** and **S2 Tables** and the **Supporting Information**. The ophthalmological examinations included measurements of IOP and optic nerve head assessment. All 19 studies contributed to the IOP mega/meta-analysis, 18 to the VCDR and 16 to the cup area and disc area mega/meta-analysis .Studies performed genomic imputation using 1000 Genomes phase 1 reference samples . Study-specific quality control can be found in the **Supporting Information**. All studies were performed with the approval of their local medical ethics committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Statistical analysis

In the IOP analysis, individuals who underwent IOP-lowering laser or surgery were removed from the analysis; in individuals receiving IOP-lowering medication, the IOP value was multiplied by 1.3 to

estimate a pre-medication IOP value[47]. The mean IOP, VCDR, cup area, and disc area of both eyes was used for the analyses. SNPs with MAF < 0.01 and imputation quality scores <0.3 (proper-info of IMPUTE) or R2<0.3 (MACH) were removed from the analyses. Each individual study performed a linear regression between each endophenotype (IOP, VCDR, cup area, and disc area) and the SNPs, under the assumption of an additive model for the effect of the risk allele. Analyses were adjusted for age, sex and the first five principal components (for population-based studies) or family structure (for family-based studies).

We performed an inverse variance weighted fixed-effect meta-analysis with METAL software[48]. P values for heterogeneity were calculated by using the Cochran's Q-test for heterogeneity. SNPs with a p-value for heterogeneity <0.001 were removed from the results, as well as SNPs only present in three studies. We used the 'genomic control' option in METAL to correct the standard error of each individual study for estimated genomic inflation. In the meta-analyses of individuals with European ancestry, a p-value $<5.0 \times 10^{-8}$ (the genome-wide threshold of association) was considered significant. In the second stage, these genome-wide significant SNPs were validated in individuals with Asian ancestry, and in this look-up a p value <0.05 was considered significant. Finally, in the meta-analysis of individuals with European and Asian ancestry a p-value of $<5.0 \times 10^{-8}$ was considered significant. In total, we identified 75 independent SNPs across different genomic regions for all the traits together. Therefore, the significance level after Bonferroni correction in the meta-analysis of POAG cohorts was = 6.67×10^{-4} (0.05 / 75 independent SNPs). To estimate the common effect size of the top SNPs associated with IOP, optic disc parameters and their effect in the look-up in the POAG cohorts a random-effect meta-analysis was performed using plink[49] http://pngu.mgh.harvard.edu/purcell/plink/ parameter *--meta-analysis*. Manhattan, regional and forest plots were made using R[50] and LocusZoom[51].

Gene-based test using VEGAS

A gene-based test was performed using the VEGAS2 software[52], with a 50kb gene boundary. We used the parameter '-top 100' (default) to perform gene-based tests. This parameter considers association test statistics of all variants mapped to a gene to compute gene-based test statistics. The 1000 Genomes European and Asian populations were used as a reference to calculate LD for European and Asian ancestry data respectively. After calculation of gene-based test statistics for Asian and European ancestry populations separately, meta-analyses were conducted using Fisher's method for combining p-values.

Functional characterization, expression data, zebrafish and gene-set enrichment

We investigated for evidence of regulatory functions of associated loci HaploReg version 2[53] and Regulomedb version 1.1[54]. We investigated the expression of the associated genes using NCBI's UniGene[17] and The Ocular Tissue Database[18]. We also investigated the expression of *cdkn1a* in a *six6b* knockdown zebrafish and used DEPICT to investigate gene-set enrichment. More information about these analyses can be found in the **Supporting Information**.

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Conflict of interest

Dr. Pasquale has been a paid speaker for Allergan. He also served as a nonpaid consultant to Novartis and a paid consultant to Bausch + Lomb. He has received support to travel to the Exfoliation Glaucoma Think Tank Meeting in NYC by the Glaucoma Foundation.

Dr. Jonas: Consultant for MundiPharma Co.; Allergan Inc.; Merck Sharp & Dohme Co., Inc.; Alimera Co.; Boehringer Ingelheim Co., Sanofi Co., Pfizer Co.; Patent holder with CellMed AG, Alzenau, Germany and with University of Heidelberg / Germany

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